

Cutaneous exposure to hypoxia does not affect skin perfusion in humans

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Short title: Exposure of human skin to hypoxia

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Abstract

Aim:

Experiments have indicated that skin perfusion in mice is sensitive to reductions in environmental O₂ availability. Specifically, a reduction in skin-surface PO₂ attenuates transcutaneous O₂ diffusion, and hence epidermal O₂ supply. In response, epidermal HIF-1 α expression increases and facilitates initial cutaneous vasoconstriction and subsequent nitric oxide-dependent vasodilation. Here, we investigated whether the same mechanism exists in humans.

Methods:

In a first experiment, eight males rested twice for eight hours in a hypobaric chamber. Once, barometric pressure was reduced by 50%, while systemic oxygenation was preserved by O₂-enriched (42%) breathing gas (Hypoxia_{skin}), and once barometric pressure and inspired O₂ fraction were normal (Control₁). In a second experiment, nine males rested for eight hours with both forearms wrapped in plastic bags. O₂ was expelled from one bag by nitrogen flushing (Anoxia_{skin}), whereas the other bag was flushed with air (Control₂). In both experiments, skin blood flux was assessed by laser Doppler on the dorsal forearm, and HIF-1 α expression was determined by immunohistochemical staining in forearm skin biopsies.

Results:

Skin blood flux during Hypoxia_{skin} and Anoxia_{skin} remained similar to the corresponding Control trial (p=0.67 and p=0.81). Immunohistochemically stained epidermal HIF-1 α was detected on 8.2 \pm 6.1 and 5.3 \pm 5.7% of the analyzed area during Hypoxia_{skin} and Control₁ (p=0.30) and on 2.3 \pm 1.8 and 2.4 \pm 1.8% during Anoxia_{skin} and Control₂ (p=0.90), respectively.

49 *Conclusion:*

50 Reductions in skin-surface PO₂ do not affect skin perfusion in humans. The unchanged epidermal HIF-1α
51 expression suggests that epidermal O₂ homeostasis was not disturbed by Hypoxia_{skin}/Anoxia_{skin},
52 potentially due to compensatory increases in arterial O₂ extraction.

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54 **Key words:** Altitude, HIF-1, Nitric oxide, Skin blood flow, Vasoconstriction, Vasodilation

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Introduction

Systemic hypoxia induces mild vasodilation in the non-glabrous skin of humans (Leuenberger et al., 1999, Minson, 2003, Simmons et al., 2007, Weisbrod et al., 2001). In hypoxic environments, this effect may be modified by a direct vasomotor response to the reduced oxygen tension (PO_2) on the skin-surface. The O_2 demand of the human skin to a depth of ~ 0.4 mm is almost exclusively covered by transcutaneous O_2 diffusion, which is driven by the transcutaneous PO_2 gradient and hence decreases in hypoxic environments (Stucker et al., 2002). In mice, the resulting reduction in O_2 supply to epidermal cells seems to facilitate the stabilization of hypoxia inducible factor-1 α (HIF-1 α) (Boutin et al., 2008, Hamanaka et al., 2016). Epidermal HIF-1 α , in turn, appears to induce a bipartite cutaneous vasomotor response, consisting of initial vasoconstriction and subsequent nitric oxide (NO)-mediated vasodilation. In the animal model, the resulting changes in skin blood flow had important systemic implications since they influenced arterial pressure, and modulated the erythropoietin response to systemic hypoxia by channeling arterial O_2 delivery first towards, and then away from kidneys and liver (Boutin et al., 2008). Whether a reduction in skin-surface PO_2 has a similar effect on skin perfusion in humans is barely explored. Rasmussen et al. (2012) observed similar increases in skin blood flow during either exposure to hypobaric hypoxia or inhalation of a hypoxic gas mixture through a mouthpiece, i.e. with a normoxic PO_2 on the skin-surface. Nevertheless, systemic hypoxia facilitated pronounced (~ 120 %) increases in skin blood flow in both conditions, which could have masked a vasomotor response to the reduced skin-surface PO_2 in hypobaric hypoxia.

In the present study, we aimed to isolate a potential skin blood flow response to reductions in skin-surface PO_2 from the cutaneous vasodilatory effect of systemic hypoxia. Two experiments were conducted: In the first experiment, eight subjects were exposed for eight hours to hypobaric hypoxia, while the inspired O_2 fraction was increased to preserve systemic oxygenation (Hypoxia_{skin}). Based on animal experiments (Boutin et al., 2008), we hypothesized Hypoxia_{skin} to first reduce and subsequently

80 increase skin blood flow. We further expected this response to be accompanied by increased epidermal
81 HIF-1 α expression and, in the later phase of exposure, circulating NO. In the second experiment, we
82 increased the stimulus by exposing one forearm of nine subjects for eight hours to anoxia (Anoxia_{skin}).
83 Again, we hypothesized Anoxia_{skin} to first reduce and subsequently increase skin blood flow as well as
84 enhance epidermal HIF-1 α expression.

85

Materials and Methods

This study was approved by the Human Ethics Committee of Stockholm (ref 2015-315-31-4) and conducted in accordance with the declaration of Helsinki.

Subjects

Eight healthy males (27 ± 8 yrs, 182 ± 8 cm, 76.1 ± 7.8 kg) were included as study subjects in the Hypoxia_{skin} experiment, and nine healthy males (28 ± 5 yrs, 183 ± 5 cm, 87.9 ± 16.2 kg) in the Anoxia_{skin} experiment. All subjects gave written informed consent to participation. None had travelled to altitudes $> 1,000$ m during the four weeks preceding the experiments.

Hypoxia_{skin} experiment

The protocol of the Hypoxia_{skin} study is summarized in figure 1. Subjects reported to the laboratory on two days, separated by at least one week, wearing a short-sleeved T-shirt and shorts. After insertion of a catheter into an antecubital vein, subjects were placed semi-recumbent in a hypobaric chamber, in which ambient air was controlled at thermoneutral temperature ($\sim 27^\circ$ C). Following instrumentation (~ 20 min), baseline measurements of the variables specified below were performed. Thereafter, the barometric pressure was, on one day, reduced by $\sim 50\%$ (to 380 mmHg, corresponding to $\sim 5,500$ m altitude) for Hypoxia_{skin}, and maintained on the other day (Control₁). The order of Hypoxia_{skin} and Control₁ was randomized between subjects. To blind the subjects, barometric pressure was repeatedly slightly increased and decreased at the onset of both trials. Subjects wore a face mask that was connected on the inspiratory side to a Douglas bag. During Hypoxia_{skin}, this Douglas bag was filled with a hyperoxic gas mixture (42 % O₂ in N₂) so that inspired PO₂ remained normal. During Control₁ the Douglas

bag was filled with normal air. Both breathing gases were bubbled through a water container for humidification before entering the Douglas bag. Throughout both trials, capillary oxyhaemoglobin saturation was continuously monitored by pulse oximetry (Radical-7, Masimo®, Irvine, CA, USA) on the subjects' earlobes.

When the final barometric pressure was reached, subjects remained still in the semi-recumbent position for eight hours, while watching movies. To assess the efficiency of the blinding process, they filled out a questionnaire immediately after the final barometric pressure was reached, as well as after four and eight hours of exposure, reporting whether they believed that barometric pressure was reduced or not. After four hours, a sandwich was provided. To avoid inspiration of chamber air, subjects removed the masks only to take a bite and put it back on for chewing and swallowing. Drinking water was provided ad libitum throughout the day employing the same mask procedure.

After eight hours, a circular skin biopsy with a diameter of 3 mm was obtained under local anesthesia (1 % lidocaine) from the dorsal forearm, using a biopsy punch (Miltex Inc., York, PA, USA). Samples were mounted in an embedding compound (Tissue-Tek O.C.T., Sakura Finetek, Alphen aan den Rijn, the Netherlands), and immediately frozen on dry ice. Subsequently, the barometric pressure was restored (if applicable), again, performing repeated increases and decreases in both conditions.

Measurements

The following measurements were conducted during baseline, after 10 min, and thereafter at every hour of exposure: Changes in skin blood flux were measured at a rate of 10 Hz on the dorsal side of the forearm by laser Doppler flowmetry (VMS-LDF2, Moor Instruments, Axminster, UK) using an optic probe (VP1/7, Moor Instruments). To examine whether Hypoxia_{skin} differently affects glabrous skin, we also monitored skin blood flux on the tip of the index finger of the same arm. Due to the variability of the microvasculature, Laser Doppler assessed skin blood flux depends on the sampling location (Obeid et al.,

1990). Accordingly, the sensors were stabilized with a flexible probe (PH1-V2, Moore) that was firmly connected to the skin with double-sided adhesive tape and were not moved throughout the entire exposure. Although only the data collected at the measurement periods specified above were used for the analysis, skin blood flux was monitored continuously throughout the exposure in order to detect deteriorations in signal quality. Laser Doppler signal stability over extended periods of continuous measurement has previously been confirmed (Sundberg, 1984). Both laser Doppler probes were calibrated before each trial against Brownian motion with a standardized colloidal suspension of polystyrene microspheres.

Arterial pressure was measured at a sampling rate of 200 Hz using the volume-clamp method (Finometer PRO, Finapres Medical Systems B.V., Amsterdam, the Netherlands), with the pressure cuff placed around the middle phalanx of the middle finger, and the reference pressure transducer placed at the vertical level of the heart. The pressure cuff was removed after each measurement period for subject comfort and the Finometer re-calibrated at the onset of the next measurement period. Heart rate was derived from the arterial pressure curves as the inverse of the inter-beat interval. An index of cardiac stroke volume was determined by a three-element model of arterial input impedance (Modelflow, Finometer PRO) incorporating age, sex, height, and weight from the arterial pressure waveform (Wesseling et al., 1993).

Cardiac output was calculated by multiplication of stroke volume with heart rate.

All these measurements were performed over a period of 10 min at each measurement time point (except after 10 min of exposure, where the measurement period was only 5 min). Unfiltered raw data was visually inspected for artefacts and then averaged over the respective measurement period for analysis.

Venous blood sampling

During baseline, as well as after one, four and eight hours of exposure, we collected 15 ml of venous blood. Nitrite (NO_2^-) concentration was assessed as a marker for circulating NO (Lauer et al., 2001) by chemiluminescence (NOA 280i, GE Analytical Instruments, Boulder, CO, USA) in plasma obtained from these samples. Concentrations of erythropoietin and vascular endothelial growth factor (VEGF), a recognized HIF-1 α target, were quantified in serum by sandwich ELISA (Human Quantikine ELISA kit, DVE00 and DEP00 respectively, R&D systems, Minneapolis, MN, USA). All samples were assayed in duplicate by a blinded investigator. The techniques and materials used in this analysis were in accordance with the protocol provided by the company. Optical density was quantified on a VersaMax microplate reader using Softmax Pro 6.3 Software (Molecular Devices, Wokingham, UK).

Epidermal HIF-1 α expression

Frozen sections (8 μm) of skin biopsies were placed on glass slides and fixed in ice-cold acetone for 10 minutes, followed by incubation with 1 % hydrogen peroxide (H_2O_2) in phosphate-buffered saline (PBS) for inactivation of endogenous peroxidase activity. After incubation with PBS containing 3 % bovine serum albumin (BSA) for 1 h at room temperature, a murine anti-human HIF-1 α antibody (NB 100-131, Novus Biologicals, Littleton, CO, USA) diluted 1:100 in PBS with 1 % BSA was applied to the sections and incubated over night at 4 °C. For negative control stainings, the primary antibody was substituted with 1 % BSA. Resultant antigen-antibody-enzyme complex was visualised using diaminobenzidine (DAB) as chromogenic substrate for peroxidase. As a control, nuclei were counterstained with haematoxylin. The sections were mounted with Faramount Aqueous Mounting Medium (DAKO A/S). 40x pictures of dermal and epidermal regions were taken with an Axio Imager M2 (Zeiss, Oberkochen, Germany), and stained areas were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The stained fraction of the analysed area was used for the quantification of epidermal HIF-1 α expression (Rizzardi et al., 2012, Cowburn et al., 2013).

Anoxia_{skin} experiment

Subjects reported to the laboratory on one day and were placed in the same position as in the Hypoxia_{skin} experiment. They were dressed in shorts and T-shirt, and the room air temperature was controlled at $\sim 27^{\circ}$ C. A plastic bag was placed over each forearm with an opening at the bottom at wrist level, so that the hands were free. The bag was loosely tightened around the arm at elbow and wrist levels with elastic tape. A small hole was cut into each bag, through which a plastic hose was inserted. The holes were then sealed around the hose with tape. One hose was connected to a cylinder containing pure N₂ (Anoxia_{skin}), and the other to a cylinder containing normal air (Control₂). Gas flow from both cylinders was then started and regulated so that there was a slight overpressure in each bag, with gas flowing out at the openings at the elbows and wrists. The continuous inflow of N₂ rapidly washed out any residual O₂ from the Anoxia_{skin} bag, as confirmed on several occasions (Datex Normocap 200 Oxy, Instrumentarium Corp., Helsinki, Finland). After initiation of the gas flow, subjects remained still while watching movies. A sandwich was provided after four hours. After eight hours, another hole was cut into each plastic bag, through which skin biopsies were obtained as described above. During the biopsy procedure, the gas flow into the bags was increased to prevent inflow of room air. After the biopsies were obtained, the gas flow was stopped and the plastic bags removed.

Measurements

In the Anoxia_{skin} experiment, we measured changes in skin blood flux on the dorsal side of both forearms with laser Doppler after each hour of exposure. Epidermal HIF-1 α expression was assessed in the biopsies as described above.

203 *Statistics*

204 Statistical analyses were performed using Statistica 8.0 (StatSoft, Tulsa, OK, USA). All data are reported
205 as mean \pm SD. Normal distribution of the data was confirmed by Shapiro-Wilks tests. Subsequently, a
206 two-way (condition \times time) general linear model repeated measures ANOVA was used to examine the
207 differences in all variables. Mauchly's test was conducted to assess for sphericity, and the Greenhouse-
208 Geisser ϵ correction was used to adjust the degrees of freedom, when the assumption of sphericity was
209 not satisfied. When ANOVA revealed significant F-ratio for interaction, pairwise comparisons were
210 performed with Tukey honestly significant difference *post hoc* test to assess differences between single
211 measurement points. The alpha level of significance was set a priori at 0.05.

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Results

Hypoxia_{skin} experiment

Methodological evaluation

Continuous pulse oximetry confirmed normal ($\sim 97\%$) capillary oxyhemoglobin saturation throughout both the Hypoxia_{skin} and Control₁ trials, hence excluding inspiration of hypoxic air during Hypoxia_{skin}. Subjects replied 48 times whether they believed that barometric pressure was reduced or not (three times per subject and trial). They were indecisive 38 times, guessed correctly twice and incorrectly 8 times, hence confirming efficient blinding.

Skin blood flux

At baseline before the start of Hypoxia_{skin} and Control₁, skin blood flux on the forearm was 137 ± 89 and 123 ± 61 arbitrary units (AU; $p = 0.60$), whereas skin blood flux on the fingertip was 376 ± 85 and 353 ± 125 AU ($p = 0.74$), respectively. Changes in skin blood flux from these baseline values are illustrated in figure 2; they were similar in Hypoxia_{skin} and Control₁ for both the forearm ($p = 0.67$) and the fingertip ($p = 0.78$).

Epidermal HIF-1 α expression

In two subjects, the biopsy obtained during Hypoxia_{skin} could not be analyzed due to technical problems. Epidermal HIF-1 α expression in the other six subjects is illustrated in figure 3; no difference was observed between Hypoxia_{skin} and Control₁ ($p = 0.30$).

Circulating NO₂⁻

In one subject, venous NO_2^- concentration considerably exceeded the expected physiological range in both conditions, and these data were hence excluded from the figure and the statistics. As illustrated in figure 4, venous NO_2^- concentration remained similar throughout Hypoxia_{skin} and Control₁ ($p = 0.34$) in the remaining seven subjects.

Systemic response

Markers of the systemic response to Hypoxia_{skin} and Control₁ are presented in table 1. For simplicity, we only present results obtained during baseline, as well as after 10 min, 1, 4 and 8 hours of exposure. Systolic ($p = 0.59$) and diastolic ($p = 0.62$) arterial pressure, cardiac stroke volume ($p = 0.69$) and cardiac output ($p = 0.34$) remained similar throughout Hypoxia_{skin} and Control₁. In contrast, HR was differently affected by Hypoxia_{skin} and Control₁ ($p < 0.009$). The subsequent post-hoc test could, however, not identify any significant differences between single measurement points.

Circulating VEGF and erythropoietin

VEGF and erythropoietin concentrations measured in venous blood are summarized in table 2; both remained similar throughout exposure to Hypoxia_{skin} and Control₁ (VEGF, $p = 0.27$ and erythropoietin, $p = 0.76$).

Anoxia_{skin} experiment

Figure 5 illustrates the changes from baseline for forearm skin blood flux throughout Anoxia_{skin} and Control₂. No significant difference between Anoxia_{skin} and Control₂ was detected ($p = 0.81$).

Figure 6 presents the individual responses of epidermal HIF-1 α expression, where no differences were observed between Anoxia_{skin} and Control₂ ($p = 0.90$).

Discussion

Our main findings are that neither a reduction in skin-surface PO_2 nor the absence of O_2 at the skin-surface had an effect on skin blood flow, or on epidermal HIF-1 α expression. Furthermore, Hypoxia_{skin} had no effect on NO metabolism.

Our hypotheses were based on a study exposing mice with epidermis-specific knockout of the HIF-1 α gene to hypoxia (Boutin et al., 2008). Comparison to wild-type mice indicated that epidermal HIF-1 α expression in hypoxia facilitates initial constriction and subsequent dilation of the cutaneous vasculature. Reducing the inspired PO_2 , while preserving the PO_2 surrounding the body, subsequently revealed that epidermal HIF-1 α expression and the resulting cutaneous vasomotor responses occur as a consequence of the low PO_2 on the skin-surface, rather than in the arterial blood. To test whether reductions in skin-surface PO_2 exert a similar effect in humans, we first conducted the Hypoxia_{skin} experiment, which involved a reduction in skin-surface PO_2 that may occur during normal human life. Exposure of the whole body surface to the reduced PO_2 furthermore allowed investigating the systemic effects of a potential skin blood flow response. Since we observed no effect of Hypoxia_{skin}, we conducted the Anoxia_{skin} experiment, in which the stimulus was maximized by complete removal of O_2 from the skin-surface. Together, the results of the two experiments strongly contradict that the human cutaneous vasculature is responsive to reductions in skin-surface PO_2 . The unchanged epidermal HIF-1 α expression furthermore implies that neither Hypoxia_{skin} nor Anoxia_{skin} disturbed epidermal O_2 homeostasis. This is surprising, since PO_2 -driven transcutaneous O_2 diffusion represents the principal O_2 source for epidermal cells (Stucker et al., 2002). A potential explanation could be that arterial O_2 delivery replaced transcutaneous O_2 diffusion during Hypoxia_{skin} and Anoxia_{skin}. To distinguish any potential effect of a reduced skin-surface PO_2 on skin blood flow from the cutaneous vasodilatory response to systemic hypoxia, we reduced the PO_2 on the skin-surface while preserving arterial PO_2 . Accordingly, if

transcutaneous O₂ diffusion decreased or even ceased during Hypoxia_{skin} and Anoxia_{skin}, the resulting reduction in epidermal PO₂ enhanced the PO₂ gradient from the capillary blood, which may have accelerated O₂ diffusion from the blood into the epidermis. A balance between O₂ diffusion into the epidermis from the blood and from the skin-surface is supported by the observation that experimentally-induced changes in arterial O₂ delivery to the skin lead to opposing changes in transcutaneous O₂ diffusion (Stucker et al., 2000). In this context, the thermoneutral environment in our study could have played a role since in a cold environment thermoregulatory cutaneous vasoconstriction (Elstad et al., 2014) might have reduced arterial O₂ delivery to the skin. In contrast to our study, the animals in the mouse study were breathing a hypoxic gas mixture when the PO₂ on the skin-surface was manipulated (Boutin et al., 2008). The low arterial PO₂ may have prevented a compensatory increase in arterial O₂ extraction when transcutaneous O₂ diffusion decreased, leading to more pronounced disturbance of epidermal O₂ homeostasis.

Taken together, our results contradict the notion that a decrease in skin-surface PO₂ independently affects skin blood flow in humans. Whether the combination of a reduced PO₂ in the inspired air and on the skin-surface stimulate epidermal HIF-1 α expression remains to be determined, although the consequence on skin blood flow may be difficult to isolate from the cutaneous vascular response to systemic hypoxia in such a setup.

The cutaneous vasomotor response to a reduction in skin-surface PO₂ in mice was bipartite, consisting of vasoconstriction within the first five hours and subsequent vasodilation (Boutin et al., 2008). While the mechanism underlying the vasoconstriction remains speculative, the vasodilation was linked to stimulation of NO synthase expression in skin cells (Cowburn et al., 2013). We did not detect an effect of Hypoxia_{skin} on circulating NO₂⁻; however, since epidermal HIF-1 α remained unchanged, its proposed regulatory role regarding cutaneous NO metabolism is neither supported, nor challenged. Interestingly, epidermal HIF-1 α -induced stimulation of NO synthase expression and the resulting cutaneous

vasodilation in mice was associated with a reduction in arterial pressure (Boutin et al., 2008, Cowburn et al., 2013). If epidermal HIF-1 α stimulates NO metabolism also in humans, a similar reduction in arterial pressure could be expected given the extensive vascularization of the human skin and the important role of NO in the regulation of cutaneous vascular tone (Clough, 1999). A negative correlation between epidermal HIF-1 α expression and arterial pressure was indeed observed in humans ranging from normo- to hypertensive (Cowburn et al., 2013). These augural findings are not expanded by the present results, again, since epidermal HIF-1 α was unaffected by Hypoxia_{skin}. Nevertheless, given the possible implication for the pathophysiology of hypertension, the potential role of epidermal HIF-1 α in arterial pressure regulation deserves further attention.

We acknowledge that during both Hypoxia_{skin} and Anoxia_{skin}, the forearm skin blood flux tended to be slightly lower than in the respective Control trials. The small subject number hereby constitutes a study limitation as it provides us with insufficient statistical power to rule out a type II error. Still, even if the skin blood flux data from the two experiments are pooled, there is no significant difference between Hypoxia_{skin}/Anoxa_{skin} and Control₁/Control₂ ($p = 0.16$), and there was also no effect of Hypoxia_{skin} on the perfusion of the glabrous skin of the fingertip. Furthermore, none of the variables that were hypothesized to mediate the cutaneous vasomotor response to reductions in skin-surface PO₂ were affected. Finally, no systemic consequences of Hypoxia_{skin} were observed, indicating that even if a slight cutaneous vasomotor response to reductions in skin-surface PO₂ was overlooked, it would have been too minor to have notable physiological consequences.

There are further limitations to this study: First, since only male subjects were included, an effect of reductions in skin surface PO₂ cannot be ruled out in females. Second, due to the short half-life of NO in blood (Liu et al., 1998), we used NO₂⁻ as marker for circulating NO. Nevertheless, NO₂⁻ accurately reflects changes in NO synthase activity (Lauer et al., 2001) and our measurement method has both, high accuracy and precision (Nagababu and Rifkind, 2007). Third, we cannot exclude that Hypoxia_{skin} or

Anoxia_{skin} affected a variable that was not monitored. Indeed, preliminary findings suggest that anoxia on the skin surface of humans might affect cerebral blood flow regulation and autonomic control (Pucci et al., 2012), although this remains to be confirmed with more direct measurement methods.

In conclusion, the present study does not support that the skin perfusion of healthy men responds to changes in skin-surface PO₂. Since neither Hypoxia_{skin} nor Anoxia_{skin} affected epidermal HIF-1 α expression, a different experimental model will have to be used to investigate whether epidermal HIF-1 α plays a role in the regulation of NO metabolism, skin perfusion and arterial pressure in humans.

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344 **Conflict of interest**

345 None of the authors has a conflict of interest to declare.

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Tables

Table 1: Systemic response to Hypoxia_{skin}

| | | Baseline | 10 min | 1 hour | 4 hours | 8 hours |
|-----------------------------------|-------------------------|-----------|-----------|-----------|-----------|-----------|
| Systolic arterial | Control ₁ | 126 ± 15 | 126 ± 15 | 128 ± 11 | 133 ± 7 | 138 ± 7 |
| pressure (mmHg) | Hypoxia _{skin} | 121 ± 7 | 128 ± 12 | 123 ± 8 | 130 ± 12 | 131 ± 10 |
| Diastolic arterial | Control ₁ | 71 ± 11 | 71 ± 7 | 72 ± 6 | 71 ± 3 | 75 ± 6 |
| pressure (mmHg) | Hypoxia _{skin} | 70 ± 9 | 73 ± 9 | 69 ± 4 | 69 ± 7 | 71 ± 8 |
| Heart rate | Control ₁ | 64 ± 8 | 59 ± 5 | 57 ± 4 | 57 ± 4 | 61 ± 4 |
| (beats min⁻¹) * | Hypoxia _{skin} | 60 ± 8 | 60 ± 8 | 58 ± 8 | 58 ± 7 | 63 ± 7 |
| Stroke volume | Control ₁ | 113 ± 10 | 114 ± 14 | 115 ± 13 | 122 ± 16 | 111 ± 13 |
| (ml) | Hypoxia _{skin} | 113 ± 14 | 110 ± 17 | 108 ± 17 | 119 ± 18 | 113 ± 17 |
| Cardiac output | Control ₁ | 7.2 ± 1.1 | 6.6 ± 0.7 | 6.6 ± 1.0 | 6.9 ± 1.1 | 6.8 ± 0.9 |
| (l min⁻¹) | Hypoxia _{skin} | 6.7 ± 1.1 | 6.1 ± 1.3 | 6.2 ± 1.1 | 6.8 ± 0.9 | 7.1 ± 1.2 |

Measurements were obtained before (Baseline), after 10 min and then after every hour of exposure.

Results obtained after 2, 3, 5, 6 and 7 hours are omitted for simplicity. Values are means ± SD. *p < 0.05 for comparison between the responses to Control₁ and Hypoxia_{skin}. No significant differences between single measurement points were identified by post-hoc testing.

407 **Table 2:** Effect of Hypoxia_{skin} on circulating erythropoietin and vascular endothelial growth factor

| | | Baseline | 1 hour | 4 hours | 8 hours |
|------------------------------|-------------------------|-------------|-------------|-------------|-------------|
| Erythropoietin | Control ₁ | 10.9 ± 2.5 | 11.2 ± 2.5 | 11.3 ± 3.0 | 10.2 ± 3.3 |
| (U l⁻¹) | Hypoxia _{skin} | 12.7 ± 4.4 | 13.1 ± 4.4 | 13.1 ± 5.7 | 11.3 ± 4.7 |
| VEGF | Control ₁ | 36.6 ± 13.8 | 45.6 ± 12.7 | 47.8 ± 18.8 | 43.4 ± 15.4 |
| (µMol l⁻¹) | Hypoxia _{skin} | 44.9 ± 14.9 | 41.4 ± 13.3 | 45.6 ± 11.9 | 41.0 ± 4.3 |

408 Erythropoietin and vascular endothelial growth factor (VEGF) concentrations were measured in venous
 409 blood that was obtained before (Baseline) as well as after 1, 4 and 8 hours of exposure. Values are
 410 means ± SD. No significant differences were observed between the responses to Control₁ and
 411 Hypoxia_{skin}.

412

Legends to figures

Figure 1: Protocol of the Hypoxia_{skin} study.

P_B, barometric pressure; F_IO₂, O₂ fraction in the inspired gas mixture; P_IO₂, inspired partial pressure of O₂.

Figure 2: Effect of Hypoxia_{skin} on skin blood flux.

Skin blood flux was measured in arbitrary units (AU) before (Baseline, BL), after 10 min, and then after every hour of exposure on the dorsal forearm (triangles) and on the index fingertip (circles). Results are presented as changes from the BL values. Data points represent means \pm SD. No significant differences were observed between Control₁ and Hypoxia_{skin}.

Figure 3: Effect of Hypoxia_{skin} on epidermal HIF-1 α expression.

HIF-1 α expression was assessed by immunohistochemical staining in skin biopsies obtained from the dorsal forearm. The biopsies of two subjects could not be analysed for technical reasons and the data points illustrate the individual results for the remaining 6 subjects. Short, horizontal lines represent the average values during Control₁ and Hypoxia_{skin}, respectively. No significant difference was observed between Control₁ and Hypoxia_{skin}.

Figure 4: Effect of Hypoxia_{skin} on circulating NO₂⁻.

NO₂⁻ was measured as a marker for NO before (Baseline, BL), as well as after 1, 4 and 8 hours of exposure. Data points represent means \pm SD. No significant differences were observed between Control₁ and Hypoxia_{skin}.

436

437 **Figure 5:** Effect of Anoxia_{Skin} on skin blood flux.

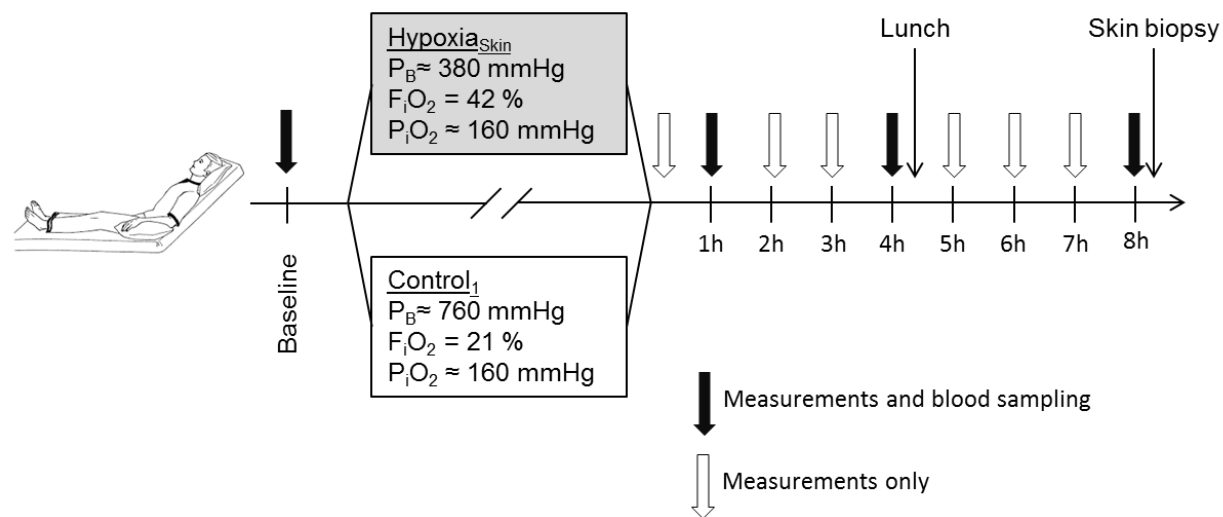
438 Skin blood flux was measured in arbitrary units (AU) before (Baseline, BL), and then after every hour of
439 exposure on the dorsal forearm. Results are presented as changes from the BL values. Data points
440 represent means \pm SD. No significant differences were observed between Control₂ and Anoxia_{Skin}.

441

442 **Figure 6:** Effect of Anoxia_{Skin} on epidermal HIF-1 α expression.

443 HIF-1 α expression was assessed by immunohistochemical staining in skin biopsies obtained from the
444 dorsal forearm. Data points illustrate individual results and short, horizontal lines represent the average
445 values during Control₂ and Anoxia_{Skin}, respectively. No significant difference was observed between
446 Control₂ and Anoxia_{Skin}.

447

448 **Figure 1:**

449

450

Figure 2:

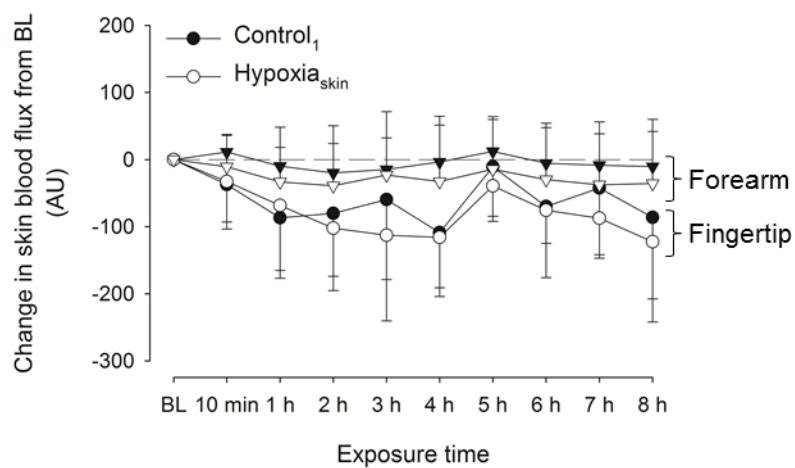


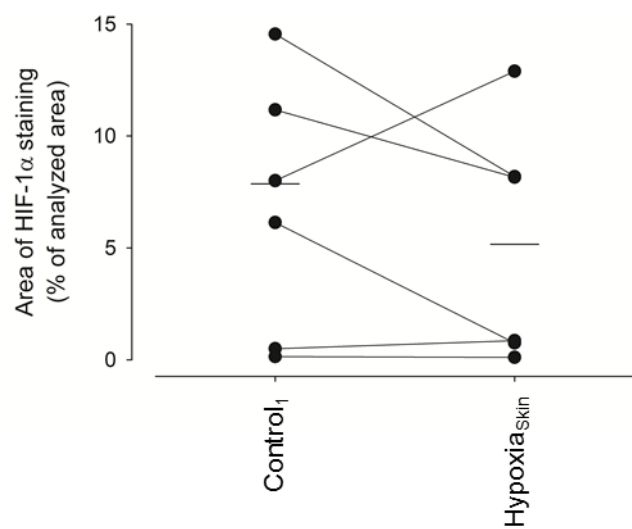
Figure 3:

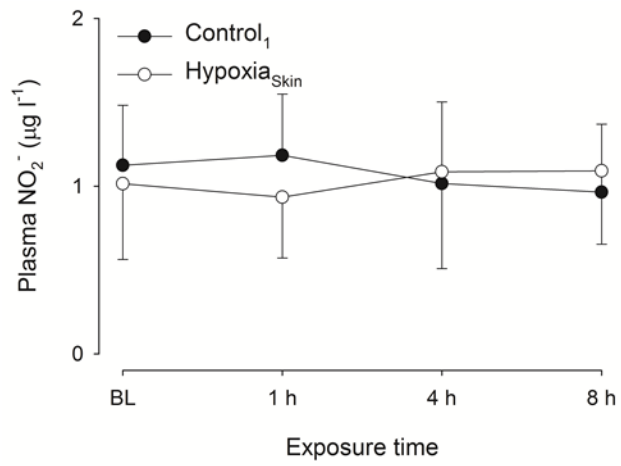
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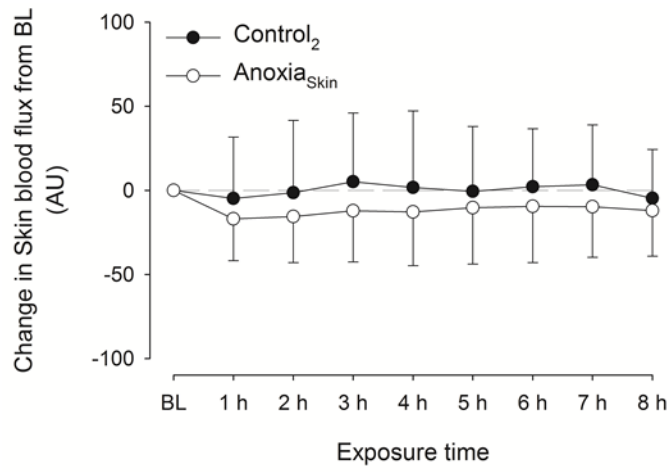
Figure 5:

Figure 6:

